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Anti-Tumour Activity of *Digitalis purpurea* L. subsp. *heywoodii*

Original Paper

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Abstract

Recent research has shown the anticancer effects of digitalis compounds suggesting their possible use in medical oncology. Four extracts obtained from the leaves of *Digitalis purpurea* subsp. *heywoodii* have been assessed for cytotoxic activity against three human cancer cell lines, using the SRB assay. All of them showed high cytotoxicity, producing IC_{50} values in the 0.78 – 15 μg/mL range with the methanolic extract being the most active, in non toxic concentrations. Steroid glycosides (gitoxigenin derivatives) were detected in this methanolic extract. Gitoxigenin and gitoxin were evaluated in the SRB assay using the three human cancer cell lines, showing IC_{50} values in the 0.13 – 2.8 μM range, with the renal adenocarcinoma cancer cell line (TK-10) being the most sensitive one. Morphological apoptosis evaluation of the methanolic extract and both compounds on the TK-10 cell line showed that their cytotoxicity was mediated by an apoptotic effect. Finally, possible mechanisms involved in apoptosis induction by digitalis compounds are discussed.

Key words
Digitalis purpurea subsp. *heywoodii* · Scrophulariaceae · gitoxin · gitoxigenin · cancer · apoptosis

Introduction

Digitalis has been used as a cardiac drug for more than 200 years mainly for the treatment of cardiac insufficiency and some types of cardiac arrhythmias. In the 1960 s a clear inhibition of malignant cells by cardiac glycosides *in vitro* was reported [1], but the conclusion from subsequent short-term animal experiments was that probably toxic doses would be needed to see interesting anticancer effects in humans [2]. However, there is a great difference in susceptibility for cardiac glycosides in different species indicating that one cannot extrapolate the results from animal models into humans [3]. Accordingly, in 1979 it was observed that breast cancer cells from women on digitalis had more benign characteristics than cancer cells from control patients not on digitalis [4], [5] and that five years after a mastectomy, the recurrence among patients not taking digitalis was 9.6 times that in patients on digitalis [6]. Surprisingly, these results did not trigger much research activity at that time to further evaluate a possible benefit of cardiac glycosides in cancer treatment. Recently, however, digitalis in non-toxic concentrations has been shown to induce apoptosis in different malignant cell lines [7] and an interesting feature seems to be that malignant cells in general are more susceptible to the effects of digitalis than normal cells [8]. A recent study, of 9271 patients, shows a relationship between high plasma concentrations of digitoxin and a lower risk for leukaemia/lymphoma and for cancer of the kidney/urinary tract [9]. These observations seems to support the idea that further research is needed to see whether cardiac glycosides can be used as anti-tumour drugs.

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With these precedents and as part of our continuing research for plants having anti-tumour activity, we have evaluated four extracts, obtained from the leaves of *Digitalis purpurea* subsp. *heywoodii*, for cytotoxic activity against three human cancer cell lines: TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma), using the SRB assay.

Materials and Methods

Plant material

The leaves of *Digitalis purpurea* L subsp. *heywoodii* (Scrophulariaceae) were collected in July 1994 in Badajoz (Spain), and dried at room temperature. A voucher specimen was authenticated by Dr. J. García and is deposited in the herbarium at the Department of Botany, Faculty of Pharmacy, University of Seville (Se 128793).

Drugs tested

Air-dried, powdered leaves of *Digitalis purpurea* subsp. *heywoodii* were extracted successively with hexane, chloroform, ethyl acetate, methanol and water, to furnish the corresponding extracts. The hexanic (2.1 %), ethyl acetate (1.8 %), methanolic (25.5 %), and aqueous (32 %) extracts were used for the cytotoxic assays (the dry weight of each extract per 100 g of leaves).

Positive controls etoposide (> 99 %), gitoxin (95 %), and gitoxigenin (> 99.5 %) were purchased from Sigma Chemical Co. Stock solutions of these drugs were dissolved in dimethyl sulfoxide the concentration of which in the tested dilutions was less than 1 % (vol/vol).

Determination of steroid glycosides content

We have used the Kedde reaction, a colorimetric technique that allows the determination of unsaturated pentacyclic lactones (present in cardiac glycosides from *Digitalis*) by using 3,5-dinitrobenzoic acid. Based on a gitoxigenin standard, we have determined the percentages of cardiac glycosides in the extracts, expressed as the mean ± SEM.

Assay for cytotoxic activity

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. They were kindly provided by Dr. G. Cragg, Department of NCI, Maryland, USA. The human tumour cytotoxicities were determined following protocols established by the National Cancer Institute, National Institute of Health [10].

Testing procedure and data processing: For the assay, cells were detached with 0.1 % trypsin-EDTA (Sigma) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final concentrations of 15×10^4, 5×10^4 and 100×10^4 cells/mL for TK-10, MCF-7 and UACC-62, respectively. 100 μL/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h the cells were treated with the serial concentrations of compounds or extracts. They were initially dissolved in an amount of 100 % DMSO (10 mM) and further diluted in medium to produce 5 concentrations. 100 μL/well of each concentration was added to the plates to obtain final concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M for the compounds and 250, 25, 2.5, 0.25 and 0.025 μg/mL for the extracts. The DMSO concentration for the tested dilutions was not greater than 0.25 % (vol/vol), the same as in solvent control wells. The final volume in each well was 200 μL. The plates were incubated for 48 h.

Sulphorhodamine B method: This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. After incubating for 48 h, adherent cell cultures were fixed *in situ* by adding 50 μL of cold 50 % (wt/vol) trichloroacetic acid (TCA) and incubating for 60 minutes at 4 °C. The supernatant is then discarded, and the plates are washed five times with deionised water and dried. One hundred μL of SRB solution (0.4 % wt/vol in 1 % acetic acid) are added to each microtiter well and the culture incubated for 30 minutes at room temperature. Unbound SRB was removed by washing five times with 1 % acetic acid. Then the plates were air-dried. Bound stain is solubilised with Tris buffer, and the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 492 nm. At the end, IC_{50} values were calculated and at least three independent experiments were carried out for each compound or extract. Data are given as the mean ± SEM.

Apoptotic index

For morphological evaluation, in order to discriminate between cells in the process of apoptosis, living, and dead (either necrotic or apoptotic), a fluorescent microscopy assay previously reported by us [11] was employed. The procedure consists of the use of a mixture of three fluorescent dyes in phosphate-buffered salt solution (PBS) at pH 7.4 : 5 μg/mL of the non-permeant dye propidium iodide (PI), 15 μg/mL of fluorescein diacetate (FDA) and 2 μg/mL of Hoechst 33342. After a 48 h drug exposure, drug removal, and a period of 5 days in fresh medium (time necessary for TK-10 cells to induce high levels of apoptosis after drug removal) 2 mL aliquots from each sample were used from the cultures and 40 μL of the fluorescent mix were added. After 5 min incubation in CO_2 atmosphere at 37 °C, the cell suspension was transferred to centrifugation tubes and washed with medium and resuspended in 1 mL medium. Samples were dropped onto slides; for observation and quantification a fluorescent microscope (Olympus, Vanox AHBT3) equipped with ultraviolet light filters was used. At least 500 cells were counted for each compound and concentration and three independent experiments were carried out. Data are given as the mean ± SEM.

Results

The results shown in Table **1** summarise the cytotoxic activity of the four extracts from *Digitalis purpurea* subsp. *heywoodii*, as well as gitoxin and gitoxigenin, on TK-10, MCF-7 and UACC-62 cell lines. The antineoplasic agent etoposide was taken as positive control for comparison with the tested extracts and compounds. The four extracts showed cytotoxic activity on the three cell lines at the recommended NCI (USA) doses in a similar fashion to that observed for the positive control etoposide, with the methanolic extract being the most cytotoxic one. According to the NCI, all these extracts, except for the hexanic extract on TK-

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Table **1** Extracts (μg/mL) and compounds (μM) concentration required to inhibit cell growth by 50% (IC_{50})

Extract/compound	*TK-10*	*MCF-7*	*UACC-62*
Hexanic extract	15.00 ± 1.00	3.65 ± 1.00	2.80 ± 0.68
Ethyl acetate extract	0.83 ± 0.04	0.85 ± 0.25	1.00 ± 0.15
Methanolic extract	0.78 ± 0.07	0.76 ± 0.20	1.09 ± 0.10
Aqueous extract	0.83 ± 0.09	0.88 ± 0.10	1.00 ± 0.11
Gitoxin	0.13 ± 0.02	0.25 ± 0.03	0.36 ± 0.01
Gitoxigenin	0.415 ± 0.03	1.80 ± 0.32	2.83 ± 0.17
Etoposide	9.95 ± 0.08	0.87 ± 0.21	1.13 ± 0.21

Data are given as the mean of at least three independent experiments ± SEM.

10 cell line, can be considered cytotoxic in the three cancer cell lines, because their cytotoxic parameter, IC_{50}, is less than 10 μg/mL The less cytotoxic extract is the less polar one, indicating that if you increase the polarity of the extract, the cytotoxicity is also increased. We have determined quantitative data concerning cardiac glycosides in the three polar extracts The similar percentage of cardiac glycosides (MeOH extract: 2.23 ± 0.20; aqueous extract: 2.38 ± 0.17; ethyl Acetate extract: 1.76 ± 0.48) in the extracts can be considered in accordance with their similar cytotoxicities shown in our cancer cell lines. In addition, these data suggest that the percentages of cardiac glycosides in the leaves of the plant might be around 0.5%, which can be considered in accordance with the literature.

Gitoxigenin derivatives were detected by preparative thin layer chromatography in the same methanolic extract in a previous work by Navarro et al. [12]. Consequently, gitoxigenin and gitoxin (the glycoside of gitoxigenin) were selected for evaluation in the three human cancer cell lines, showing IC_{50} values in the 0.13 – 2.8 μM range, being even more active than the antineoplastic agent etoposide. The glycoside gitoxin was clearly more active than its aglycone, gitoxigenin, indicating that sugar moieties are important for the activity.

In order to know if digitalis cytotoxicity was due to an apoptotic or necrotic effect, cell death was measured using a three-fluorescent dye method as reported elsewhere [11]. This methodology allows us to clearly distinguish cells that are either alive (green fluorescence), apoptotic (blue) or necrotic (red). We chose the methanolic extract on the basis of the toxicity date [12], the digitalis compounds (gitoxin and gitoxigenin), along with etoposide (VP-16) as positive control, to assess any possible induction of apoptotic cell death. The concentrations assessed were those previously shown as able to result in an inhibition of growth corresponding to 50 percent (IC_{50}) according to the SRB assay. At these concentrations it was clear that the methanolic extract, as well as gitoxin and gitoxigenin induced an apoptotic effect. Apoptosis induction by gitoxin was higher than by gitoxigenin, suggesting that not only is the sugar moiety necessary for a high cytotoxicity, but also for an increased apoptotic cell death. However, when we increased the concentration of both compounds, apoptosis decreased and necrotic cell death increased.

Discussion

A recent study carried out by Haux and coworkers [9] in which the incidence of different cancers in 9271 patients taking a digitalis compound (digitoxin) was evaluated. An internal dose-response analysis revealed a relationship between high plasma concentrations of digitoxin and a lower risk for leukaemia/lymphoma and for cancer of the kidney/urinary tract. In fact, patients with plasma concentrations of digitoxin within the 16 – 22 ng/mL range showed 3.3 times less incidence of these cancers than patients with plasma concentrations less than 16 ng/mL. The lack of toxicity for patients of digitoxin at these concentrations appears to sustain experimental data showing that the most interesting feature about digitalis is that malignant cells, in general, seem more susceptible to its effects than normal

Table **2** Morphological apoptosis evaluation on TK-10 cancer cell line

	Concentration	*% normal cells*	*% apoptotic cells*	*% necrotic cells*
Control	–	72.8 ± 2.8	22.4 ± 4.5	4.8 ±2.9
Methanolic extract	1 μg/mL	33.7 ± 5.3	58.4 ± 8.7	7.9 ± 4.9
Gitoxin	0.13 μM	31.2 ± 4.3	64.1 ± 7.7	4.7 ± 3.5
Gitoxin	1 μM	31.1 ± 7.2	48.5 ± 6.7	20.4 ± 7.5
Gitoxigenin	0.42 μM	46.7 ± 3.8	43.1 ± 5.1	10.2 ± 4.3
Gitoxigenin	1 μM	29.0 ± 6.4	31.7 ± 5.8	39.3 ± 9.6
Etoposide	10 μM	7.7 ± 1.8	28.6 ± 2.3	63.7 ± 3.6

Data are given as the mean of at least three independent experiments ± SEM.

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ones [8]. The high activity of our digitalis compounds in the human cancer cell lines assayed might be considered in accordance with the anti-tumour effect shown by therapeutic concentrations of cardiac glycosides observed in patients with breast carcinoma and cancer of the kidney/urinary tract [4], [5], [6], [9], suggesting digitalis sensitivity for cancer cells.

The present report supports other investigations showing that apoptosis induction is a major effect of digitalis on several types of tumour cells [7], [8], [13], [14]. Ca^{2+} has a pivotal role in the apoptotic process. Increased intracellular Ca^{2+} concentration may start apoptosis by itself, and is also a step in several cascades leading to apoptosis after receptor interaction [15]. Therefore, increased intracellular Ca^{2+} derived from Na^+/K^+-ATPase inhibition seems a mechanism of action involved in apoptosis induction by digitalis. Until now, focus has been on the Na^+/K^+-ATPase as the primary target for the anticancer effects of the digitalis compounds. However, now that we know that apoptosis induction is a major effect of digitalis, it seems fruitful to explore mechanisms other than the Na^+/K^+-ATPase inhibition [8]. In this way, there are several facts that suggest that DNA topoisomerases might be involved in the anticancer activity of digitalis. Firstly, irinotecan is a topoisomerase poison used in therapeutic treatment as an anticancer agent which possesses a lactone moiety, such as that present in digitalis compounds, seemingly this structural feature is crucial for its anticancer effects [16]. Secondly, different digitalis compounds have shown a radiosensitising effect on malignant cancer cell lines but not on normal ones [8], [17] and, interestingly, malfunction of topoisomerases has been proposed to be involved in the radiosensitisation processes [18]. In addition, poisoning of topoisomerases is frequently associated with apoptosis [19], and the ability of digitalis to induce apoptosis in cancer cells is clear (see references above). Finally, the levels of topoisomerases in cancer cells are generally higher than in normal ones [20]. Therefore, drugs able to interact with topoisomerases may show selectivity for cancer cells. Experiments ongoing in our laboratory seem to support these theoretical speculations. Although more experiments are needed, our preliminary results show that some digitalis compounds target human DNA topoisomerases.

In conclusion, since recent research shows the possible usefulness of digitalis in medical oncology, the present report has shown the anticancer activity of *Digitalis purpurea* L. subsp. *heywoodii* on three human cancer cell lines. A methanolic extract from the leaves of this plant was able to induce apoptosis on a resistant cancer cell line at non-toxic concentrations. Digitalis compounds gitoxin and gitoxigenin have been shown, for the first time, to induce apoptosis on a human cancer cell line, supporting several works showing that apoptosis is a major effect of other digitalis compounds. Finally, we have discussed the possible mechanisms of action involved in apoptosis induced by these compounds and, based on preliminary results, we suggest that DNA topoisomerases might be involved in the anticancer effects of digitalis.

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